

Protective effect of bilobalide against nitric oxide-induced neurotoxicity in PC12 cells¹

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KEY WORDS PC12 cells; SIN-1; neurotoxins; neurons; cell survival; lipid peroxidation; neuroprotective agents; antioxidants; bilobalide

ABSTRACT

AIM: To examine the effects of bilobalide on nitric oxide-induced neurotoxicity in pheochromocytoma-derived PC12 cells (PC12 cells). **METHODS:** PC12 cell survival was monitored by LDH release and 3-(4,5-dimethylthiazol-2-yl)-2,5-diphenyltetrazolium bromide (MTT) assays. Superoxide dismutases (SOD) and catalase (CAT) activities were measured based on their abilities to inhibit the oxidation of epinephrine by the xanthine-xanthine oxidase system or to decompose H₂O₂ respectively. The content of malondialdehyde (MDA) was measured by a fluorometric assay to indicate the lipid peroxidation. **RESULTS:** 3-Morpholinosydnonimine (SIN-1, 50–300 μmol·L⁻¹) induced PC12 cell damage. After the cells had been pretreated with 10 μmol·L⁻¹ bilobalide for 24 h, the cell viability was increased to 91% ± 30% from 52% ± 14% in SIN-1 alone group. Moreover, the activities of SOD and CAT were increased after cells were treated with bilobalide. **CONCLUSION:** The NO-induced neurotoxicity can be protected by bilobalide in PC12 cells. The bilobalide-induced increase in SOD and CAT activities may serve as one of the mechanisms underlying the neuroprotective effect of bilobalide.

INTRODUCTION

The standardized extract of *Ginkgo biloba* and its

constituents ginkgolide A, ginkgolide B, and bilobalide have neuroprotective effects^[1,2]. The platelet-activating factor (PAF) antagonistic activity was suggested to be one of mechanisms underlying the neuroprotective effect of the ginkgolides, especially ginkgolide B. However, bilobalide, the main constituent of the non-flavone fraction of the extract of *Ginkgo biloba*, does not possess any PAF-antagonistic properties. Recently, we have studied the possible mechanisms underlying the neuroprotective effect of bilobalide. We found that bilobalide protected the pheochromocytoma-derived PC12 cells (PC12 cells) from β-amyloid peptide-induced PC12 cell death and suggested that the regulatory effect of bilobalide on antioxidant enzymes might be involved in its neuroprotective effects^[3].

Nitric oxide (NO) is a reactive free radical. In the central nervous system (CNS), NO plays important roles in neurotransmitter release and re-uptake, synaptic plasticity, and regulation of gene expression. However, excessive production of NO following a pathologic insult can lead to neurotoxicity. Evidence has shown that NO plays important roles in neurotoxicity associated with a variety of neurologic disorders, including stroke, Parkinson's disease, and Alzheimer's disease^[4]. Peroxynitrite (ONOO⁻), produced by reaction of NO with superoxide (O₂⁻), has been proposed to mediate the toxic actions caused by NO^[5]. ONOO⁻ may inhibit mitochondrial function and then lead to more free radical formation. NO/ONOO⁻ may also activate a futile cycle of DNA damage followed by poly(ADP-ribose) polymerase (PARP) activation which depletes cells of their energy stores, ultimately leading to cell death^[4]. Additionally, the conjugate acid of peroxynitrite, peroxynitrous acid (ONOOH), and/or its decomposition products, ie, ·OH and nitrogen dioxide (·NO₂), can initiate lipid peroxidation that also elicits neurotoxicity^[6]. 3-Morpholinosydnonimine (SIN-1) is the active metabolite of the vasodilator molsidomine. It has frequently been used as a method to generate ONOO⁻ continuously in solutions

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where it slowly decomposes to release both NO and superoxide and thereby produces ONOO⁻[7]. The aim of the present study was to find out whether bilobalide could protect PC12 cells from SIN-1-induced neurotoxicity.

MATERIAL AND METHODS

Cell culture PC12 cells were cultured at 37 °C in Dubecco's modified Eagles's medium (DMEM) containing 10 % heat-inactivated fetal calf serum in a humidified 5 % CO₂ atmosphere. Cells were used when in exponential growth.

Drugs Bilobalide, provided by Professor Zhong-Liang CHEN (Shanghai Institute of Materia Medica, Chinese Academy of Sciences, China), was dissolved in dimethylsulfoxide (Me₂SO). The concentration of dimethylsulfoxide in the final incubation mixture was 0.1 % (v/v). At this concentration, the solvent did not show any effect on the cell viability or antioxidant enzyme activities in the cells.

LDH release and MTT assays LDH release and MTT were measured in the same cultures. LDH activity in the extracellular medium was quantified as described previously^[8]. Briefly, phosphate-buffered saline containing sodium pyruvate 0.1 g · L⁻¹ and β-NADH 0.2 g · L⁻¹ was added to 50 μL of the medium sample. The decrease in the optical density at 340 nm was monitored for 5 min; 0.001 unit optical density decrease per min was equivalent to 1 LDH unit. The MTT assay was performed on intact cells modifying a previously described method^[9]. In brief, MTT (0.5 g · L⁻¹) was added to each well and then the culture was incubated for 4 h at 37 °C. After incubation, the medium was then separated from the wells, and 100 μL of dimethylsulfoxide was added into each well to dissolve the formazan product. The optical density at 540 nm was then measured using a plate reader (Hua Dong Electronic Co, Nanjing, China). Results were expressed as percentage of optical density in vehicle-treated control culture wells.

Levels of lipid peroxidation The content of malondialdehyde (MDA) in cultures was measured to indicate the level of lipid peroxidation as previously described^[10].

Antioxidant enzyme activity assays The superoxide dismutase (SOD) assay was based on the ability of SOD to inhibit the oxidation of epinephrine by the xanthine-xanthine oxidase system^[11]. To a 200 μL aliquot of the supernatant were added 1.48 mL of 68 mmol · L⁻¹

NaH₂PO₄ containing edetic acid 1.35 mmol · L⁻¹ (pH 7.8), 100 μL of xanthine 4 mmol · L⁻¹, and 170 μL of epinephrine 3.53 mmol · L⁻¹ (pH 11.5). After 5 min incubation at 30 °C, 50 μL of xanthine oxidase was added to the cuvette, and the optical density measured for 3 min at 320 nm. One unit of SOD activity was defined as the amount that reduced the optical density (OD) change by 50 %, and results were expressed as units per milligram of protein.

The catalase activity was measured as described previously^[11]. Cell homogenate supernatant (1.5 mL) was mixed with 18 μL of absolute ethanol and incubated on ice for 8 min, and 7.8 μL of 10 % Triton X-100 and 2.5 mL of 10 mmol · L⁻¹ phosphate buffer (pH 7.4) were added. Aliquots (0.6 mL) of the solution were added to 20-mL test tubes along with 3.57 mL of cold 6 mmol · L⁻¹ H₂O₂, and the tube was vortex-mixed. After 3 min, 0.714 mL of H₂SO₄ 3 mol · L⁻¹ and 5 mL of KMnO₄ 0.01 mol · L⁻¹ were added to the solution. After the tube was vortex-mixed, OD was measured at 480 nm within 60 s using a Spec 21 UV-Vis spectrophotometer. Quantification was based on the comparison of the tissue samples to a calibration curve of known peroxide concentrations. The activity of the enzyme was expressed as units per mg of protein, with 1 unit equivalent to 1 mmol of H₂O₂ catalyzed per min.

Statistical analysis The data for multiple comparisons was performed by ANOVA followed by Dunnett's test.

RESULTS

Bilobalide protects PC12 cells against NO toxicity The exposure of PC12 cells to SIN-1 for 20 h produced a concentration-dependent reduction in the cell viability, assayed by MTT (Fig 1) and LDH release (Fig 2). When cultures were pretreated with bilobalide 1 μmol · L⁻¹ for 24 h and then exposed to SIN-1, the cell damage was greatly attenuated (Fig 1, 2). After the cells had been pretreated with bilobalide 10 μmol · L⁻¹ for 24 h, the cell viability was increased to 91 % ± 30 % comparing with 52 % ± 14 % in SIN-1 alone group (Fig 3). Bilobalide alone at the same concentration range had no effect on the cell viability (data not shown).

The neuroprotective effect of bilobalide was time-dependent. The SIN-1-induced neurotoxicity was reduced only after the cultures were pretreated with bilobalide for at least 6 h (Fig 4).

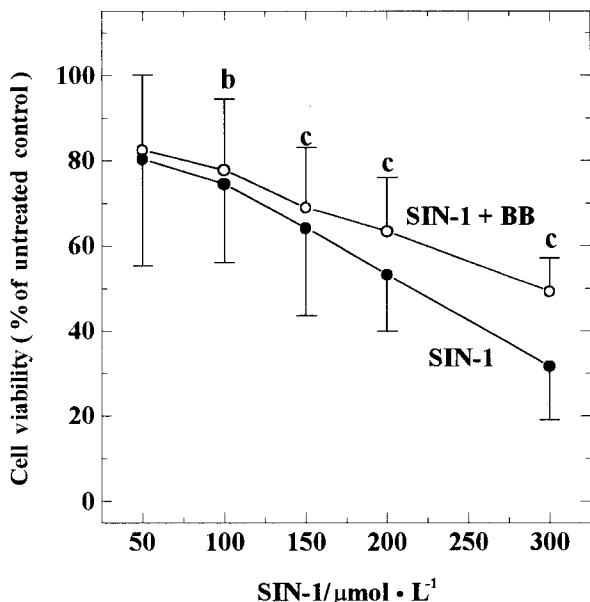


Fig 1. Effect of bilobalide on SIN-1-induced toxicity in PC12 cells. Cells were pretreated with saline or bilobalide (BB, 1 μmol · L⁻¹) for 24 h and then exposed to SIN-1 for another 20 h. Cell viability was measured by MTT assay. n = 3 experiments (4 wells in each experiment). $\bar{x} \pm s$. ^bP < 0.05, ^cP < 0.01 compared with SIN-1 alone group.

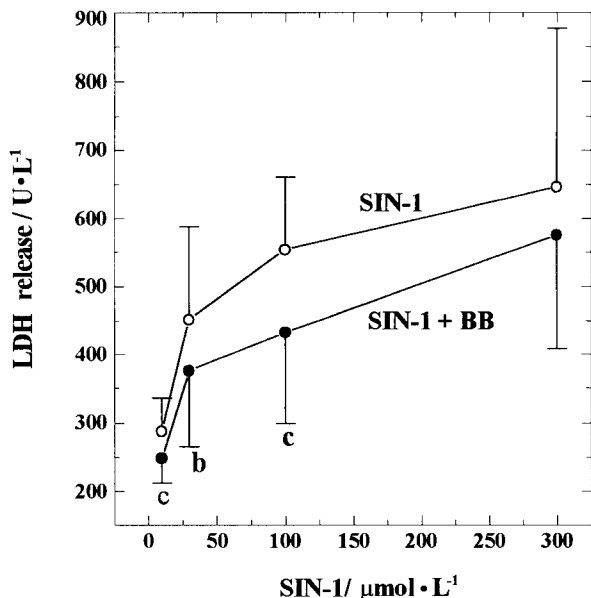


Fig 2. Effect of bilobalide on SIN-1-induced LDH release in PC12 cells. Cells were pretreated with saline or bilobalide (BB, 1 μmol · L⁻¹) for 24h and then exposed to SIN-1 for another 20 h. LDH release was measured. n = 3 experiments (4 wells in each experiment). $\bar{x} \pm s$. ^bP < 0.05, ^cP < 0.01 compared with SIN-1 alone group.

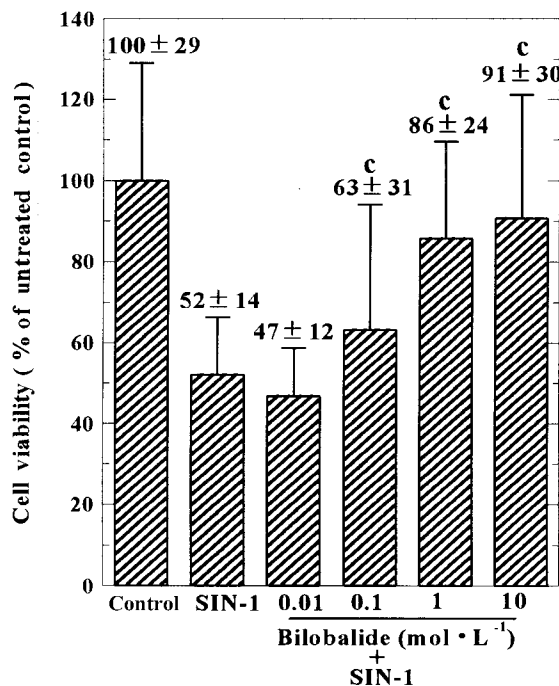


Fig 3. Effect of different concentration of bilobalide on SIN-1-induced toxicity in PC12 cells. Cells were pretreated with saline or 0.01, 0.1, 1, 10 μmol · L⁻¹ of bilobalide (BB) for 24 h and then exposed to SIN-1 (200 μmol · L⁻¹) or SIN-1 plus BB (0.01, 0.1, 1, 10 μmol · L⁻¹) for another 20 h. The cell viability was assessed by MTT assay. n = 3 experiments (4 wells in each experiment). $\bar{x} \pm s$. ^cP < 0.01 compared with SIN-1 alone group.

Bilobalide attenuates NO-induced increase of lipid peroxidation When PC12 cells were exposed to SIN-1 (10 – 200 μmol · L⁻¹) for 24 h, an increase in lipid peroxidation level was found (Tab 1). The level of lipid peroxidation increased to 300 % of the control when the cells were treated with SIN-1 (200 μmol · L⁻¹). Pretreatment with bilobalide 1 μmol · L⁻¹ had moderated, but statistically significant reduction in the lipid peroxidation induced by SIN-1.

Bilobalide increases activities of antioxidant enzymes When PC12 cells were pretreated with bilobalide for 24 h, the levels of SOD and catalase activities were increased in a dose-dependent manner. In control cultures, the basal activity of SOD was (2.9 ± 1.2) U · mg⁻¹ (protein). The SOD activity increased nearly 2 fold in PC12 cells after the cells were exposed to bilobalide 10 μmol · L⁻¹ for 24 h (Fig 5A). The catalase activity was elevated approximately 3 fold from (12 ± 4) U · g⁻¹ (protein) in control to (31 ± 10) U · g⁻¹ (protein) in bilobalide-treated cultures (Fig 5B).

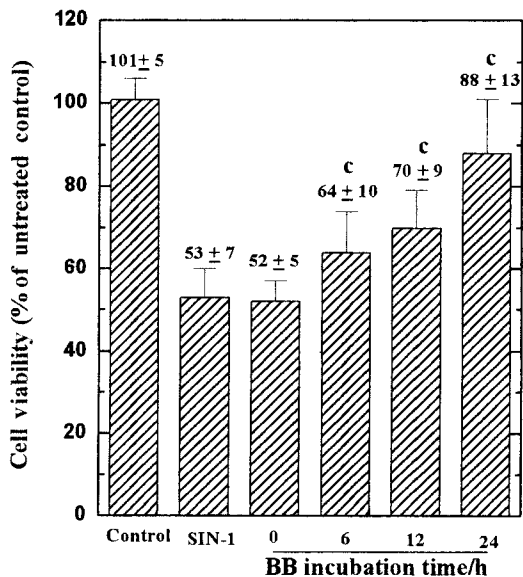


Fig 4. Time course of bilobalide effect on SIN-1-induced toxicity in PC12 cells. Cells were pretreated with saline or bilobalide (BB, 1 $\mu\text{mol} \cdot \text{L}^{-1}$) for 0–24 h and then exposed to SIN-1 (200 $\mu\text{mol} \cdot \text{M}^{-1}$) or SIN-1 plus BB (1 $\mu\text{mol} \cdot \text{L}^{-1}$) for another 20 h. The cell viability was assessed by MTT assay. $n = 3$ experiments (4 wells in each experiment). $\bar{x} \pm s$. ^c $P < 0.01$ compared with SIN-1 alone group.

Tab 1. Effect of bilobalide on SIN-1-induced increase in lipid peroxidation in PC12 cells. Cells were pretreated with saline or bilobalide (1 $\mu\text{mol} \cdot \text{L}^{-1}$) for 24 h and then exposed to SIN-1 for another 20 h. The content of MDA in the cultures was measured to indicate the lipid peroxidation. $n = 3$ experiments (4 wells in each experiment). $\bar{x} \pm s$. ^b $P < 0.05$, ^c $P < 0.01$ vs control group. ^e $P < 0.05$, ^f $P < 0.01$ vs SIN-1 alone group.

Groups	SIN-1/ $\mu\text{mol} \cdot \text{L}^{-1}$	MDA/nmol $\cdot \text{L}^{-1}$ mg protein
Saline	0	2.17 ± 0.13
Saline	10	2.8 ± 0.93 ^b
Saline	100	4.1 ± 0.74 ^c
Saline	200	6.9 ± 2.91 ^c
Bilobalide	10	2.1 ± 0.47 ^e
Bilobalide	100	2.8 ± 0.6 ^f
Bilobalide	200	4.3 ± 1.3 ^f

DISCUSSION

In the present study, we demonstrate that SIN-1-induced PC12 cell damage was attenuated by bilobalide pretreatment. Although the mechanisms sustaining the neuroprotective effect of bilobalide are not fully clarified, our results suggest that the upregulation of SOD and CAT

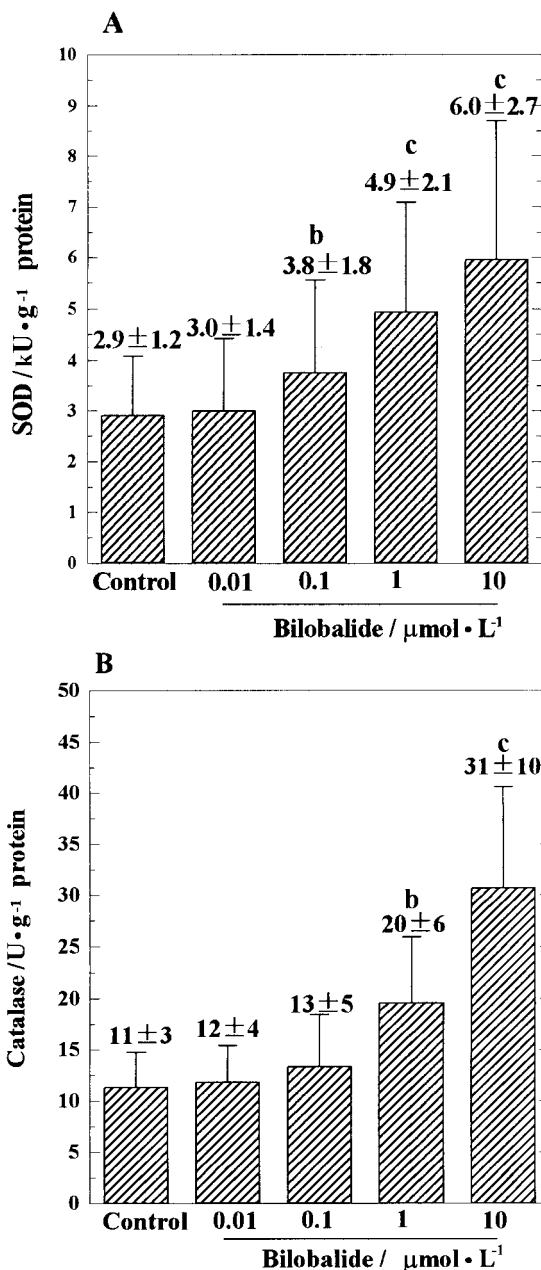


Fig 5. Effect of bilobalide on antioxidant enzyme activities in PC12 cells. Cells were treated with bilobalide (0.01, 0.1, 1, 10 $\mu\text{mol} \cdot \text{L}^{-1}$) for 24 h and then SOD (A) and CAT (B) activities were measured. $n = 3$ experiments (4 wells in each experiment). $\bar{x} \pm s$. ^b $P < 0.05$, ^c $P < 0.01$ compared with SIN-1 alone group.

activities may be involved.

There is a growing body of evidence to implicate excessive or inappropriate generation of NO in neurodegenerative disorders^[12]. Neurons, in contrast to astrocytes, appear particularly vulnerable to the action of NO and its toxic metabolite, ONOO⁻. Following a pathologic insult, astrocytes can increase NO generation, due to *de*

novo synthesis of the inducible form of nitric oxide synthase (NOS). Whilst the NO/ONOO⁻ formed may not affect astrocyte survival, these molecules may diffuse out to cause mitochondrial damage, and possibly cell death in other cells such as neurons. This has provided the rationale for designing selective NOS inhibitors for the treatment of neurodegenerative disorders.

Overexpression of endogenous antioxidant system, such as SOD, may prevent apoptotic cell death^[13]. SOD can dismutate two O²⁻ into H₂O₂ and O₂^[14] and, in the presence of CAT, can protect cells from damage induced by simultaneous generation of NO and O²⁻^[15]. Our results are consistent with this hypothesis. The anti-apoptotic effect of bilobalide has been described recently^[21]. In this study, apoptosis was induced by serum deprivation or addition of staurosporine. Both treatments lead to an increase in the production of reactive oxygen species, an effect that can be blocked by antioxidants.

Recently, several placebo-controlled, double-blind and randomized trials have confirmed that the extract of *Ginkgo biloba* was effective in mild to moderate dementia in the AD patients^[16] and was capable of stabilizing and improving the cognitive performance and the social functioning of demented patients^[17]. However, the mechanisms underlying the neuroprotective effect of this extract are not fully understood. Our results show that bilobalide, the main constituent of the non-flavone fraction of the extract of *Ginkgo biloba*, protected PC12 cells from NO-induced cell death. This may provide one of the mechanisms underlying the neuroprotective effect of the extract of *Ginkgo biloba*.

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白果内酯在 PC12 细胞中对 NO 诱导的细胞毒性的保护作用¹

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关键词 PC12 细胞; SIN-1; 神经毒素; 神经元; 细胞存活; 脂质过氧化作用; 神经保护剂; 抗氧化剂; 白果内酯

目的: 研究白果内酯在 PC12 细胞中对 NO 诱导的细

胞毒性的保护作用. 方法: 以 MTT 法及 LDH 法检测细胞存活率; 同时检测细胞的超氧化物歧化酶(SOD)、过氧化氢酶(CAT)活性及脂质过氧化水平. 结果: NO 供体 SIN-1 ($50 - 300 \mu\text{mol} \cdot \text{L}^{-1}$) 可导致 PC12 细胞死亡, 可使 PC12 细胞脂质过氧化水平升高. 白果内酯预孵育可减少 NO 诱导的细胞死亡; 可抑制脂质过氧化水平升高. 白果内酯预孵育本身可使 SOD 及 CAT 酶活性升高. 结论: 白果内酯对 NO 诱导的细胞毒性作用具保护作用, 该保护作用可能与白果内酯升高细胞内 SOD 和 CAT 的活性有关.

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